

METHODS AND COMPOSITIONS FOR DETECTION AND ANALYSIS OF
POLYNUCLEOTIDES USING LIGHT HARVESTING MULTICHROMOPHORES

Inventors:

Guillermo C. Bazan
Santa Barbara, California
Citizenship: U.S.A.

Brent S. Gaylord
Santa Barbara, California
Citizenship: U.S.A.

Assignee:

The Regents of the University of California
1111 Franklin Street, Twelfth Floor
Oakland, CA 94607-5200

Prepared By:

David W. Maher
Bingham McCutchen LLP
Three Embarcadero Center, Suite 1800
San Francisco, California 94111
(650) 849-4908

METHODS AND COMPOSITIONS FOR DETECTION AND ANALYSIS
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MULTICHROMOPHORES

CROSS REFERENCE TO RELATED APPLICATIONS

- 5 [0001] This application claims the benefit of U.S. Provisional Application Nos. 60/390,524, filed June 20, 2002 and 60/406,266, filed August 26, 2002, which are hereby incorporated by reference in their entireties.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

- 10 [0002] Work leading to this invention was performed under grant number GM62958-01 from the National Institutes of Health, grant number DMR-0097611 from the National Science Foundation and grant number N00014-98-1-0759 from the Office of Naval Research. The U.S. Government may have limited rights in this invention.

15 TECHNICAL FIELD

- [0003] This invention relates to methods, articles and compositions for the detection and analysis of polynucleotides in a sample.

BACKGROUND OF THE INVENTION

- 20 [0004] Methods permitting DNA sequence detection in real time and with high sensitivity are of great scientific and economic interest.^{1,2,3} Their applications include medical diagnostics, identification of genetic mutations, gene delivery monitoring and specific genomic techniques.⁴ Cationic organic dyes, such as ethidium bromide and thiazole orange, emit when intercalated into the grooves of double strand DNA (dsDNA),
25 and serve as direct DNA hybridization probes, but lack sequence specificity.^{5,6} Energy/electron transfer chromophore pairs for strand specific assays exist, but require chemical labeling of two nucleic acids, or dual modification of the same altered strand

(for example, molecular beacons).^{7,8} Difficulties in labeling two DNA sites result in low yields, high costs and singly labeled impurities, which lower detection sensitivity.⁹

[0005] The recent introduction of peptide nucleic acids (PNAs) has provided an opportunity for new research and diagnostic applications.^{10,11} In PNAs, the negatively charged phosphate linkages in DNA are replaced with peptidomimetic neutral amide linkages. PNA/DNA complexes form more quickly, with higher binding energies, and are more specific than analogous DNA/DNA complexes.¹² These enhanced properties result from the absence of Coulombic repulsion as occurs between negatively charged DNA strands. PNA complexes are thus more thermally stable and, by virtue of their backbone, less susceptible to biological degradation by nucleases, proteases and peptidases.^{13,14} Additionally, their general insensitivity to ionic strength and pH during hybridization provides a wider platform for DNA detection.¹⁸

[0006] There is a need in the art for methods of detecting and analyzing particular polynucleotides in a sample, and for compositions and articles of manufacture useful in such methods.

SUMMARY OF THE INVENTION

[0007] Methods, compositions and articles of manufacture for detecting and assaying a target polynucleotide in a sample are provided. A sample suspected of containing the target polynucleotide is contacted with a polycationic multichromophore and a sensor peptide nucleic acid (PNA) complementary to the target polynucleotide. The sensor PNA is conjugated to a signaling chromophore. Without wishing to be bound by theory, in the presence of target polynucleotide in the sample, the signaling chromophore is believed to be brought into proximity with the cationic multichromophore by utilizing electrostatic interactions with the backbone of the target polynucleotide which has hybridized to the sensor PNA (see Figure 1). The signaling chromophore can then acquire energy from the excited polycationic multichromophore and emit light which can be detected. The target polynucleotide can be analyzed as it naturally occurs in the sample, or can be amplified prior to or in conjunction with analysis. A solution is provided comprising reagents useful for performing the methods of the invention, as are kits containing such reagents. The methods can be used in multiplex settings where a plurality of different sensor PNAs

are used to assay for a plurality of different target polynucleotides. The methods can optionally be performed on a surface, for example using a surface-associated polycationic multichromophore; the surface can be a sensor. The methods can also be provided in homogeneous formats. The methods and articles described herein can be used as
5 alternatives to other techniques for detecting polynucleotides.

BRIEF DESCRIPTION OF THE DRAWINGS

[0008] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawings will be
10 provided by the Patent and Trademark Office upon request and payment of the necessary fee.

[0009] Figure 1 depicts the method of the invention, employing a polycationic polymer as the light-harvesting multichromophore. A sensor PNA (PNA-C*) comprising a signaling chromophore and having a base sequence complementary to the target
15 polynucleotide of interest is provided. Upon contacting the target polynucleotide in a sample, the polycationic multichromophore is brought into proximity with the signaling chromophore by virtue of electrostatic interactions with the target polynucleotide. Excitation of the multichromophore then produces light emission from the signaling chromophore.

20 [00010] Figure 2 presents the respective absorption (green and orange) and emission (blue and red) spectra of polymer 1 (see below) and the sensor peptide nucleic acid PNA-C*. Excitation was performed at 380 and 480 nm for 1 and PNA-C*, respectively.

[00011] Figure 3 presents the emission spectra of PNA-C* in the presence of complementary (red) and non-complementary (black) DNA by excitation of polymer 1.
25 PNA-C* and the respective DNA were added together in water at pH=5.5. The spectra are normalized with respect to the emission of polymer 1.

[00012] Figure 4 presents the emission spectra of PNA-C* in the presence of complementary (red) and non-complementary (black) DNA by excitation of oligomer 2.
30 PNA-C* and the respective DNA were added together in water at pH=5.5. The spectra are normalized with respect to the emission of oligomer 2.

DETAILED DESCRIPTION OF THE INVENTION

[00013] Present technologies for DNA and RNA sensors (including “gene-chips” and “DNA-chips”) depend on the covalent attachment of fluorescent tags (lumophores) to single strands of DNA. Most of these sensors are forced to rely on the labeling of the
5 analyte sample, with unavoidable problems resulting from variations in the efficiency of the labeling reaction from sample to sample, requiring complex cross-calibrations. Other systems rely on the “molecular beacon” approach, requiring the attachment of lumophores and quenchers to precisely engineered sequences.

10 [00014] One method of the invention comprises contacting a sample with at least two components in a predominantly aqueous solution: (a) a light harvesting, luminescent multichromophore system such as, for example, a conjugated polymer, semiconductor quantum dot or dendritic structure that is water soluble; and (b) a sensor PNA conjugated to a luminescent signaling chromophore or “PNA-C*”. The emission of a wavelength of
15 light characteristic of the signaling chromophore-C* upon excitation of the multichromophore indicates the presence in solution of the target polynucleotide. By using multiple different sensor PNAs, each with a different base sequence and a different signaling chromophore (PNA₁-C₁^{*}, PNA₂-C₂^{*}, PNA₃-C₃^{*}, PNA₄-C₄^{*}, etc), multiple different polynucleotides can be independently detected and assayed.

20 [00015] The light harvesting chromophore and the signaling chromophore (C^{*}) are chosen so that the absorption bands of the two chromophores have minimal overlap and so that the luminescent emission spectra of the two species are at different wavelengths. When prepared in aqueous solution, the light harvesting luminescent multichromophore system is positively charged, or cationic, and is preferably polycationic (for example a
25 polycationic conjugated polyelectrolyte). Since the sensor PNA is not charged, there is minimal Coulombic interaction between the sensor PNA and the cationic light harvesting luminescent multichromophore system. Upon addition of a target polynucleotide complementary to the sequence of the sensor PNA, the target polynucleotide hybridizes with the sensor PNA. Because the target polynucleotide is negatively charged, the sensor
30 PNA associates with the polycationic multichromophore, permitting energy transfer from the polycationic multichromophore to the signaling chromophore, for example via the

Förster energy transfer mechanism. When a polynucleotide with a base sequence that is not complementary to that of the sensor PNA is added, base pair hybridization does not take place and electrostatically-mediated complexation between the multichromophore and the sensor PNA does not occur. Because the average distance between the polycationic multichromophore and the signaling chromophore is too large for effective energy transfer in the absence of such hybridization, there is little or no emission from the signaling chromophore. The overall scheme serves to detect the presence of the target polynucleotide in the test solution. Additionally, PNAs also have the ability to form triplex structures by binding to and invading dsDNA and displacing the DNA strand of the same sequence.^{15,16,17} Such sensor PNA/polycationic multichromophore platforms can be incorporated in systems for direct dsDNA detection.¹⁸

[00016] In addition to the described method, the invention provides a predominantly aqueous solution comprising at least two components; (a) a cationic multichromophore, and (b) a "sensor PNA" (PNA-C*) comprising a peptide nucleic acid conjugated to a signaling chromophore.

[00017] As demonstrated in the Examples, the optical amplification provided by a water soluble multichromophore such as a conjugated polymer can be used to detect polynucleotide hybridization to a PNA sensor. The amplification can be enhanced by using higher molecular weight water soluble conjugated polymers or other structures as the polycationic multichromophore as described herein. The invention can be provided in a homogeneous format that utilizes the ease of fluorescence detection methods and capitalizes on the enhanced hybridization behavior found in PNA-DNA interactions. The invention can be used to detect amplified target polynucleotides or, because of the large signal amplification, as a stand alone assay, without need for polynucleotide amplification.

[00018] Unique advantages of the invention over present gene-chip technology thus include circumvention of the requirement to first label each sample to be analyzed by covalent coupling of lumophores or chromophores to the polynucleotides contained in or derived from the sample prior to analysis. Those coupling methods have inherent difficulties in reproducibility of coupling efficiency and result in the need for cross-calibration from sample to sample.

[00019] The inventions described herein are useful for any assay in which a sample can be interrogated regarding a target polynucleotide. Typical assays involve determining the presence of the target polynucleotide in the sample or its relative amount, or the assays may be quantitative or semi-quantitative.

5 [00020] The methods of the invention can all be performed in multiplex formats. A plurality of different sensor PNAs can be used to detect corresponding different target polynucleotides in a sample through the use of different signaling chromophores conjugated to the respective sensor PNAs. Multiplex methods are provided employing 2, 3, 4, 5, 10, 15, 20, 25, 50, 100, 200, 400 or more different sensor PNAs which can be
10 used simultaneously to assay for corresponding different target polynucleotides.

[00021] The methods can be performed on a substrate, as well as in solution, although the solution format is expected to be more rapid due to diffusion. Thus the assay can be performed, for example, in an array format on a substrate, which can be a sensor. This can be achieved by anchoring or otherwise incorporating an assay component onto the
15 substrate, for example the sensor polynucleotide, the polycationic multichromophore, or both. These substrates may be surfaces of glass, silicon, paper, plastic, or the surfaces of optoelectronic semiconductors (such as, but not confined to, indium-doped gallium nitride or polymeric polyanilines, etc.) employed as optoelectronic transducers. The location of a given sensor polynucleotide may be known or determinable in an array
20 format, and the array format may be microaddressable or nanoaddressable.

[00022] Before the present invention is described in further detail, it is to be understood that this invention is not limited to the particular methodology, devices, solutions or apparatuses described, as such methods, devices, solutions or apparatuses can, of course, vary. It is also to be understood that the terminology used herein is for the purpose of
25 describing particular embodiments only, and is not intended to limit the scope of the present invention.

[00023] Use of the singular forms "a," "an," and "the" include plural references unless the context clearly dictates otherwise. Thus, for example, reference to "a target polynucleotide" includes a plurality of target polynucleotides, reference to "a signaling chromophore" includes a plurality of such chromophores, reference to "a sensor PNA"
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includes a plurality of sensor PNAs, and the like. Additionally, use of specific plural references, such as "two," "three," etc., read on larger numbers of the same subject unless the context clearly dictates otherwise.

[00024] Terms such as "connected," "attached," "linked" and conjugated are used interchangeably herein and encompass direct as well as indirect connection, attachment, linkage or conjugation unless the context clearly dictates otherwise. Where a range of values is recited, it is to be understood that each intervening integer value, and each fraction thereof, between the recited upper and lower limits of that range is also specifically disclosed, along with each subrange between such values. The upper and lower limits of any range can independently be included in or excluded from the range, and each range where either, neither or both limits are included is also encompassed within the invention. Where a value being discussed has inherent limits, for example where a component can be present at a concentration of from 0 to 100%, or where the pH of an aqueous solution can range from 1 to 14, those inherent limits are specifically disclosed. Where a value is explicitly recited, it is to be understood that values which are about the same quantity or amount as the recited value are also within the scope of the invention, as are ranges based thereon. Where a combination is disclosed, each subcombination of the elements of that combination is also specifically disclosed and is within the scope of the invention. Conversely, where different elements or groups of elements are disclosed, combinations thereof are also disclosed. Where any element of an invention is disclosed as having a plurality of alternatives, examples of that invention in which each alternative is excluded singly or in any combination with the other alternatives are also hereby disclosed; more than one element of an invention can have such exclusions, and all combinations of elements having such exclusions are hereby disclosed.

[00025] Unless defined otherwise or the context clearly dictates otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods and materials are now described.

[00026] All publications mentioned herein are hereby incorporated by reference for the purpose of disclosing and describing the particular materials and methodologies for which the reference was cited. The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

DEFINITIONS

[00027] In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

[00028] The terms "polynucleotide," "oligonucleotide," "nucleic acid" and "nucleic acid molecule" are used interchangeably herein to refer to a polymeric form of nucleotides of any length, and may comprise ribonucleotides, deoxyribonucleotides, analogs thereof, or mixtures thereof. These terms refer only to the primary structure of the molecule. Thus, the terms include triple-, double- and single-stranded deoxyribonucleic acid ("DNA"), as well as triple-, double- and single-stranded ribonucleic acid ("RNA"). It also includes modified, for example by alkylation and/or by capping, and unmodified forms of the polynucleotide.

[00029] Whether modified or unmodified, the polymeric target nucleotide must have a polyanionic backbone, preferably a sugar-phosphate background, of sufficient negative charge to electrostatically interact with the polycationic multichromophore in the methods described herein, although other forces may additionally participate in the interaction. The sensor polynucleotide is exemplified as a peptide nucleic acid, although other uncharged polynucleotides which minimally interact with the multichromophore in the absence of target can be used. Suitable hybridization conditions for a given assay format can be determined by one of skill in the art; nonlimiting parameters which may be adjusted include concentrations of assay components, pH, salts used and their concentration, ionic strength, temperature, etc.

[00030] More particularly, the terms "polynucleotide," "oligonucleotide," "nucleic acid" and "nucleic acid molecule" include polydeoxyribonucleotides (containing 2-deoxy-D-

ribose), polyribonucleotides (containing D-ribose), including tRNA, rRNA, hRNA, and mRNA, whether spliced or unspliced, any other type of polynucleotide which is an N- or C-glycoside of a purine or pyrimidine base, and other polymers containing a phosphate or other polyanionic backbone, and other synthetic sequence-specific nucleic acid polymers providing that the polymers contain nucleobases in a configuration which allows for base pairing and base stacking, such as is found in DNA and RNA. Thus, these terms include, for example, 3'-deoxy-2',5'-DNA, oligodeoxyribonucleotide N3' P5' phosphoramidates, 2'-O-alkyl-substituted RNA, double- and single-stranded DNA, as well as double- and single-stranded RNA, and hybrids thereof including for example hybrids between DNA and RNA, and also include known types of modifications, for example, labels, alkylation, "caps," substitution of one or more of the nucleotides with an analog, internucleotide modifications such as, for example, those with negatively charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), those containing pendant moieties, such as, for example, proteins (including enzymes (e.g. nucleases), toxins, antibodies, signal peptides, poly-L-lysine, etc.), those with intercalators (e.g., acridine, psoralen, etc.), those containing chelates (of, e.g., metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators, those with modified linkages (e.g., alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotide or oligonucleotide.

[00031] It will be appreciated that, as used herein, the terms "nucleoside" and "nucleotide" will include those moieties which contain not only the known purine and pyrimidine bases, but also other heterocyclic bases which have been modified. Such modifications include methylated purines or pyrimidines, acylated purines or pyrimidines, or other heterocycles. Modified nucleosides or nucleotides can also include modifications on the sugar moiety, e.g., wherein one or more of the hydroxyl groups are replaced with halogen, aliphatic groups, or are functionalized as ethers, amines, or the like. The term "nucleotidic unit" is intended to encompass nucleosides and nucleotides.

[00032] Furthermore, modifications to nucleotidic units include rearranging, appending, substituting for or otherwise altering functional groups on the purine or pyrimidine base which form hydrogen bonds to a respective complementary pyrimidine or purine. The resultant modified nucleotidic unit optionally may form a base pair with other such modified nucleotidic units but not with A, T, C, G or U. Abasic sites may be

incorporated which do not prevent the function of the polynucleotide; preferably the polynucleotide does not comprise abasic sites. Some or all of the residues in the polynucleotide can optionally be modified in one or more ways.

[00033] Standard A-T and G-C base pairs form under conditions which allow the formation of hydrogen bonds between the N3-H and C4-oxy of thymidine and the N1 and C6-NH₂, respectively, of adenosine and between the C2-oxy, N3 and C4-NH₂, of cytidine and the C2-NH₂, N'-H and C6-oxy, respectively, of guanosine. Thus, for example, guanosine (2-amino-6-oxy-9- β -D-ribofuranosyl-purine) may be modified to form isoguanosine (2-oxy-6-amino-9- β -D-ribofuranosyl-purine). Such modification results in a nucleoside base which will no longer effectively form a standard base pair with cytosine. However, modification of cytosine (1- β -D-ribofuranosyl-2-oxy-4-amino-pyrimidine) to form isocytosine (1- β -D-ribofuranosyl-2-amino-4-oxy-pyrimidine) results in a modified nucleotide which will not effectively base pair with guanosine but will form a base pair with isoguanosine. Isocytosine is available from Sigma Chemical Co. (St. Louis, MO); isocytidine may be prepared by the method described by Switzer et al. (1993) *Biochemistry* 32:10489-10496 and references cited therein; 2'-deoxy-5-methyl-isocytidine may be prepared by the method of Tor et al. (1993) *J. Am. Chem. Soc.* 115:4461-4467 and references cited therein; and isoguanine nucleotides may be prepared using the method described by Switzer et al. (1993), *supra*, and Mantsch et al. (1993) *Biochem.* 14:5593-5601, or by the method described in U.S. Patent No. 5,780,610 to Collins et al. Other nonnatural base pairs may be synthesized by the method described in Piccirilli et al. (1990) *Nature* 343:33-37 for the synthesis of 2,6-diaminopyrimidine and its complement (1-methylpyrazolo-[4,3]pyrimidine-5,7-(4H,6H)-dione). Other such modified nucleotidic units which form unique base pairs are known, such as those described in Leach et al. (1992) *J. Am. Chem. Soc.* 114:3675-3683 and Switzer et al., *supra*.

[00034] "Complementary" or "substantially complementary" refers to the ability to hybridize or base pair between nucleotides or nucleic acids, such as, for instance, between a sensor peptide nucleic acid and a target polynucleotide. Complementary nucleotides are, generally, A and T (or A and U), or C and G. Two single-stranded polynucleotides or PNAs are said to be substantially complementary when the bases of

one strand, optimally aligned and compared and with appropriate insertions or deletions, pair with at least about 80% of the bases of the other strand, usually at least about 90% to 95%, and more preferably from about 98 to 100%.

[00035] Alternatively, substantial complementarity exists when a polynucleotide or PNA will hybridize under selective hybridization conditions to its complement. Typically, selective hybridization will occur when there is at least about 65% complementary over a stretch of at least 14 to 25 bases, preferably at least about 75%, more preferably at least about 90% complementary. See, M. Kanehisa Nucleic Acids Res. 12:203 (1984).

[00036] "Preferential binding" or "preferential hybridization" refers to the increased propensity of one polynucleotide or PNA to bind to its complement in a sample as compared to a noncomplementary polymer in the sample.

[00037] Hybridization conditions will typically include salt concentrations of less than about 1M, more usually less than about 500 mM and preferably less than about 200 mM.

In the case of hybridization between a peptide nucleic acid and a polyanionic

polynucleotide, the hybridization can be done in solutions containing little or no salt.

Hybridization temperatures can be as low as 5°C, but are typically greater than 22°C, more typically greater than about 30°C, and preferably in excess of about 37°C. Longer fragments may require higher hybridization temperatures for specific hybridization.

Other factors may affect the stringency of hybridization, including base composition and length of the complementary strands, presence of organic solvents and extent of base mismatching, and the combination of parameters used is more important than the absolute measure of any one alone. Other hybridization conditions which may be controlled include buffer type and concentration, solution pH, presence and concentration of blocking reagents to decrease background binding such as repeat sequences or blocking protein solutions, detergent type(s) and concentrations, molecules such as polymers which increase the relative concentration of the polynucleotides, metal ion(s) and their concentration(s), chelator(s) and their concentrations, and other conditions known in the art.

[00038] "Multiplexing" herein refers to an assay or other analytical method in which multiple analytes can be assayed simultaneously.

[00039] "Optional" or "optionally" means that the subsequently described event or circumstance may or may not occur, and that the description includes instances where the event or circumstance occurs and instances in which it does not.

5 THE SAMPLE

[00040] The portion of the sample comprising or suspected of comprising the target polynucleotide can be any source of biological material which comprises polynucleotides that can be obtained from a living organism directly or indirectly, including cells, tissue or fluid, and the deposits left by that organism, including viruses, mycoplasma, and
10 fossils. The sample may comprise a target polynucleotide prepared through synthetic means, in whole or in part. Typically, the sample is obtained as or dispersed in a predominantly aqueous medium. Nonlimiting examples of the sample include blood, urine, semen, milk, sputum, mucus, a buccal swab, a vaginal swab, a rectal swab, an aspirate, a needle biopsy, a section of tissue obtained for example by surgery or autopsy,
15 plasma, serum, spinal fluid, lymph fluid, the external secretions of the skin, respiratory, intestinal, and genitourinary tracts, tears, saliva, tumors, organs, samples of *in vitro* cell culture constituents (including but not limited to conditioned medium resulting from the growth of cells in cell culture medium, putatively virally infected cells, recombinant cells, and cell components), and a recombinant library comprising polynucleotide
20 sequences.

[00041] The sample can be a positive control sample which is known to contain the target polynucleotide or a surrogate therefor. A negative control sample can also be used which, although not expected to contain the target polynucleotide, is suspected of containing it (via contamination of one or more of the reagents) or another component
25 capable of producing a false positive, and is tested in order to confirm the lack of contamination by the target polynucleotide of the reagents used in a given assay, as well as to determine whether a given set of assay conditions produces false positives (a positive signal even in the absence of target polynucleotide in the sample).

[00042] The sample can be diluted, dissolved, suspended, extracted or otherwise treated
30 to solubilize and/or purify any target polynucleotide present or to render it accessible to reagents which are used in an amplification scheme or to detection reagents. Where the

sample contains cells, the cells can be lysed or permeabilized to release the polynucleotides within the cells. One step permeabilization buffers can be used to lyse cells which allow further steps to be performed directly after lysis, for example a polymerase chain reaction.

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THE TARGET POLYNUCLEOTIDE AND AMPLIFICATION PRODUCTS PRODUCED THEREFROM

[00043] The target polynucleotide can be single-stranded, double-stranded, or higher order, and can be linear or circular. Exemplary single-stranded target polynucleotides include mRNA, rRNA, tRNA, hnRNA, ssRNA or ssDNA viral genomes, although these polynucleotides may contain internally complementary sequences and significant secondary structure. Exemplary double-stranded target polynucleotides include genomic DNA, mitochondrial DNA, chloroplast DNA, dsRNA or dsDNA viral genomes, plasmids, phage, and viroids. The target polynucleotide can be prepared synthetically or purified from a biological source. The target polynucleotide may be purified to remove or diminish one or more undesired components of the sample or to concentrate the target polynucleotide. Conversely, where the target polynucleotide is too concentrated for the particular assay, the target polynucleotide may be diluted.

[00044] Following sample collection and optional nucleic acid extraction, the nucleic acid portion of the sample comprising the target polynucleotide can be subjected to one or more preparative reactions. These preparative reactions can include in vitro transcription (IVT), labeling, fragmentation, amplification and other reactions. mRNA can first be treated with reverse transcriptase and a primer to create cDNA prior to detection and/or amplification; this can be done in vitro with purified mRNA or in situ, e.g. in cells or tissues affixed to a slide. Nucleic acid amplification increases the copy number of sequences of interest such as the target polynucleotide. A variety of amplification methods are suitable for use, including the polymerase chain reaction method (PCR), the ligase chain reaction (LCR), self sustained sequence replication (3SR), nucleic acid sequence-based amplification (NASBA), the use of Q Beta replicase, reverse transcription, nick translation, and the like.

[00045] Where the target polynucleotide is single-stranded, the first cycle of amplification forms a primer extension product complementary to the target

polynucleotide. If the target polynucleotide is single-stranded RNA, a polymerase with reverse transcriptase activity is used in the first amplification to reverse transcribe the RNA to DNA, and additional amplification cycles can be performed to copy the primer extension products. The primers for a PCR must, of course, be designed to hybridize to regions in their corresponding template that will produce an amplifiable segment; thus, each primer must hybridize so that its 3' nucleotide is paired to a nucleotide in its complementary template strand that is located 3' from the 3' nucleotide of the primer used to replicate that complementary template strand in the PCR.

[00046] The target polynucleotide is typically amplified by contacting one or more strands of the target polynucleotide with a primer and a polymerase having suitable activity to extend the primer and copy the target polynucleotide to produce a full-length complementary polynucleotide or a smaller portion thereof. Any enzyme having a polymerase activity which can copy the target polynucleotide can be used, including DNA polymerases, RNA polymerases, reverse transcriptases, enzymes having more than one type of polymerase activity, and the enzyme can be thermolabile or thermostable. Mixtures of enzymes can also be used. Exemplary enzymes include: DNA polymerases such as DNA Polymerase I ("Pol I"), the Klenow fragment of Pol I, T4, T7, Sequenase® T7, Sequenase® Version 2.0 T7, *Tub*, *Taq*, *Tth*, *Pfx*, *Pfu*, *Tsp*, *Tfl*, *Tli* and *Pyrococcus sp.* GB-D DNA polymerases; RNA polymerases such as *E. coli*, SP6, T3 and T7 RNA polymerases; and reverse transcriptases such as AMV, M-MuLV, MMLV, RNase H⁻ MMLV (SuperScript®), SuperScript® II, ThermoScript®, HIV-1, and RAV2 reverse transcriptases. All of these enzymes are commercially available. Exemplary polymerases with multiple specificities include RAV2 and *Tli* (exo-) polymerases. Exemplary thermostable polymerases include *Tub*, *Taq*, *Tth*, *Pfx*, *Pfu*, *Tsp*, *Tfl*, *Tli* and *Pyrococcus sp.* GB-D DNA polymerases.

[00047] Suitable reaction conditions are chosen to permit amplification of the target polynucleotide, including pH, buffer, ionic strength, presence and concentration of one or more salts, presence and concentration of reactants and cofactors such as nucleotides and magnesium and/or other metal ions (e.g., manganese), optional cosolvents, temperature, thermal cycling profile for amplification schemes comprising a polymerase chain reaction, and may depend in part on the polymerase being used as well as the nature of

the sample. Cosolvents include formamide (typically at from about 2 to about 10 %), glycerol (typically at from about 5 to about 10 %), and DMSO (typically at from about 0.9 to about 10 %). Techniques may be used in the amplification scheme in order to minimize the production of false positives or artifacts produced during amplification.

5. These include "touchdown" PCR, hot-start techniques, use of nested primers, or designing PCR primers so that they form stem-loop structures in the event of primer-dimer formation and thus are not amplified. Techniques to accelerate PCR can be used, for example centrifugal PCR, which allows for greater convection within the sample, and comprising infrared heating steps for rapid heating and cooling of the sample. One or more cycles of amplification can be performed. An excess of one primer can be used to produce an excess of one primer extension product during PCR; preferably, the primer extension product produced in excess is the amplification product to be detected. A plurality of different primers may be used to amplify different target polynucleotides or different regions of a particular target polynucleotide within the sample.

- 15 **[00048]** Amplified target polynucleotides may be subjected to post amplification treatments. For example, in some cases, it may be desirable to fragment the target polynucleotide prior to hybridization in order to provide segments which are more readily accessible. Fragmentation of the nucleic acids can be carried out by any method producing fragments of a size useful in the assay being performed; suitable physical, chemical and enzymatic methods are known in the art.

- [00049]** An amplification reaction can be performed under conditions which allow the sensor polynucleotide to hybridize to the amplification product during at least part of an amplification cycle. When the assay is performed in this manner, real-time detection of this hybridization event can take place by monitoring for a change in light emission from the signaling chromophore that occurs upon such hybridization during the amplification scheme.

THE POLYCATIONIC MULTICHROMOPHORE

- 30 **[00050]** Light harvesting multichromophore systems have been demonstrated to be efficient light absorbers by virtue of the multiple chromophores they comprise. Examples include, but are not limited to, conjugated polymers, aggregates of conjugated

molecules, luminescent dyes attached via side chains to saturated polymers, semiconductor quantum dots and dendritic structures. For example, each repeat unit on a conjugated polymer can be considered as a contributing chromophore, quantum dots are made up of many atoms, a saturated polymer can be functionalized with many luminescent dye molecules on side chains, and dendrimers can be synthesized containing many covalently bonded individual chromophores. Attachment of chromophore assemblies onto solid supports, such as polymer beads or surfaces, can also be used for light harvesting.

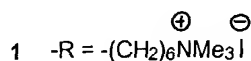
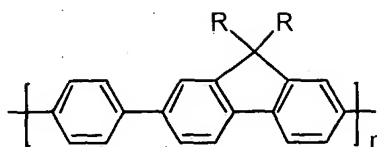
[00051] Light harvesting multichromophore systems can efficiently transfer energy to nearby luminescent species (e.g., "signaling chromophores"). Mechanisms for energy transfer include, for example, resonant energy transfer (Förster (or fluorescence) resonance energy transfer, FRET), quantum charge exchange (Dexter energy transfer) and the like. Typically, however, these energy transfer mechanisms are relatively short range; that is, close proximity of the light harvesting multichromophore system to the signaling chromophore is required for efficient energy transfer. Under conditions for efficient energy transfer, amplification of the emission from the signaling chromophore occurs when the number of individual chromophores in the light harvesting multichromophore system is large; that is, the emission from the signaling chromophore is more intense when the incident light (the "pump light") is at a wavelength which is absorbed by the light harvesting multichromophore system than when the signaling chromophore is directly excited by the pump light.

[00052] Conjugated polymers (CPs) are characterized by a delocalized electronic structure and can be used as highly responsive optical reporters for chemical and biological targets.^{19,20} Because the effective conjugation length is substantially shorter than the length of the polymer chain, the backbone contains a large number of conjugated segments in close proximity. Thus, conjugated polymers are efficient for light harvesting and enable optical amplification via Förster energy transfer.²¹ Water-soluble CPs show exceptional fluorescence quenching efficiencies in the presence of oppositely charged acceptors and are of particular interest for transduction of biological recognition events.^{22,23}

[00053] Spontaneous interpolymer complexation between cationic polyelectrolytes and DNA has been described and is largely the result of cooperative electrostatic forces.^{24,25,26} Hydrophobic interactions between aromatic polymer units and DNA bases were also recently recognized.^{27,28} The free energy of polyelectrolyte/DNA interactions is controlled by the structure of the participating species used in conjunction with solution variables such as pH, ionic strength, and temperature.²⁹ The strength and specificity of these interactions has recently been coordinated to recognize the tertiary structure of plasmid DNA.³⁰

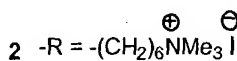
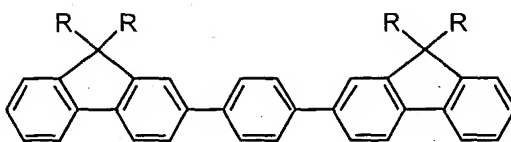
[00054] The multichromophores used in the present invention are polycationic so that they can interact with a target polynucleotide electrostatically and thereby bring a signaling chromophore on an uncharged sensor PNA into energy-receiving proximity by virtue of hybridization between the sensor PNA and the target polynucleotide. Any polycationic multichromophore that can absorb light and transfer energy to a signaling chromophore on a sensor PNA can be used in the methods described. Exemplary multichromophores which can be used include conjugated polymers (which includes oligomers), saturated polymers or dendrimers incorporating multiple chromophores in any viable manner, and semiconductor nanocrystals (SCNCs). The conjugated polymers, saturated polymers and dendrimers can be prepared to incorporate multiple cationic species or can be derivatized to render them polycationic after synthesis; semiconductor nanocrystals can be rendered polycationic by addition of cationic species to their surface.

[00055] In a preferred embodiment, a conjugated polymer is used as the polycationic multichromophore. A specific example is shown in structure 1 where the cationic water soluble conjugated polymer is poly((9,9-bis(6'-N,N,N-trimethylammonium)-hexyl)-fluorene phenylene) with iodide counteranions (denoted in the following as polymer 1).²³ The particular size of this polymer is not critical, so long as it is able to absorb light and transfer energy to signaling chromophores brought into proximity. Typical values of "n" fall within the range of two to about 100,000. This specific molecular structure is not critical; any water soluble cationic conjugated polymer with relatively high luminescence quantum efficiency can be used.



[00056] Water soluble conjugated oligomers can also be used as the polycationic multichromophore. An example of such a water soluble, cationic, luminescent conjugated oligomer with iodide counterions is shown below (denoted herein as oligomer

5 2):



[00057] Although the smaller oligomer 2 does not display the large signal amplification characteristic of a high molecular weight polymer, such smaller molecules are useful to deconvolute structure property relationships, which are difficult to determine with the inherent polydispersity and batch-to-batch variations found in polymers. Further, in aqueous media oligomers such as 2 are more soluble than their polymeric counterparts, and hydrophobic interactions with neutral PNA are expected to be less important for 2 than for polymer structures. Assemblies of oligomers may thus be desired for specific applications.

THE SENSOR PNA

[00058] A sensor PNA is provided that is complementary to the target polynucleotide to be assayed, and has a predetermined sequence. The sensor PNA can be branched, multimeric or circular, but is typically linear, and can contain nonnatural bases. The molecular structures of PNAs are well known. PNAs can be prepared with any desired sequence of bases. Chemical methods for attaching the signaling chromophore to the

sensor PNA are well known.¹⁰ Specific sensor PNA structures, including structures conjugated to chromophores, can be custom-made using commercial sources or chemically synthesized.

5 THE SIGNALING CHROMOPHORE

[00059] Chromophores useful in the inventions described herein include any substance which can absorb energy from a polycationic multichromophore in an appropriate solution and emit light. For multiplexed assays, a plurality of different signaling chromophores can be used with detectably different emission spectra. The chromophore
10 can be a lumophore or a fluorophore. Typical fluorophores include fluorescent dyes, semiconductor nanocrystals, lanthanide chelates, and green fluorescent protein.

[00060] Exemplary fluorescent dyes include fluorescein, 6-FAM, rhodamine, Texas Red, tetramethylrhodamine, a carboxyrhodamine, carboxyrhodamine 6G, carboxyrhodol, carboxyrhodamine 110, Cascade Blue, Cascade Yellow, coumarin, Cy2®, Cy3®,
15 Cy3.5®, Cy5®, Cy5.5®, Cy-Chrome, phycoerythrin, PerCP (peridinin chlorophyll-a Protein), PerCP-Cy5.5, JOE (6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein), NED, ROX (5-(and-6)-carboxy-X-rhodamine), HEX, Lucifer Yellow, Marina Blue, Oregon Green 488, Oregon Green 500, Oregon Green 514, Alexa Fluor® 350, Alexa Fluor® 430, Alexa Fluor® 488, Alexa Fluor® 532, Alexa Fluor® 546, Alexa Fluor® 568, Alexa
20 Fluor® 594, Alexa Fluor® 633, Alexa Fluor® 647, Alexa Fluor® 660, Alexa Fluor® 680, 7-amino-4-methylcoumarin-3-acetic acid, BODIPY® FL, BODIPY® FL-Br₂, BODIPY® 530/550, BODIPY® 558/568, BODIPY® 564/570, BODIPY® 576/589, BODIPY® 581/591, BODIPY® 630/650, BODIPY® 650/665, BODIPY® R6G, BODIPY® TMR, BODIPY® TR, conjugates thereof, and combinations thereof.

25 Exemplary lanthanide chelates include europium chelates, terbium chelates and samarium chelates.

[00061] A wide variety of fluorescent semiconductor nanocrystals ("SCNCs") are known in the art; methods of producing and utilizing semiconductor nanocrystals are described in: PCT Publ. No. WO 99/26299 published May 27, 1999, inventors Bawendi
30 et al.; USPN 5,990,479 issued Nov. 23, 1999 to Weiss et al.; and Bruchez et al., Science 281:2013, 1998. Semiconductor nanocrystals can be obtained with very narrow emission

bands with well-defined peak emission wavelengths, allowing for a large number of different SCNCs to be used as signaling chromophores in the same assay, optionally in combination with other non-SCNC types of signaling chromophores.

[00062] The term "green fluorescent protein" refers to both native *Aequorea* green fluorescent protein and mutated versions that have been identified as exhibiting altered fluorescence characteristics, including altered excitation and emission maxima, as well as excitation and emission spectra of different shapes (Delgrave, S. et al. (1995) Bio/Technology 13:151-154; Heim, R. et al. (1994) Proc. Natl. Acad. Sci. USA 91:12501-12504; Heim, R. et al. (1995) Nature 373:663-664). Delgrave et al. isolated mutants of cloned *Aequorea victoria* GFP that had red-shifted excitation spectra. Bio/Technology 13:151-154 (1995). Heim, R. et al. reported a mutant (Tyr66 to His) having a blue fluorescence (Proc. Natl. Acad. Sci. (1994) USA 91:12501-12504).

THE SUBSTRATE

[00063] The substrate can comprise a wide range of material, either biological, nonbiological, organic, inorganic, or a combination of any of these. For example, the substrate may be a polymerized Langmuir Blodgett film, functionalized glass, Si, Ge, GaAs, GaP, SiO₂, SiN₄, modified silicon, or any one of a wide variety of gels or polymers such as (poly)tetrafluoroethylene, (poly)vinylidenedifluoride, polystyrene, cross-linked polystyrene, polyacrylic, polylactic acid, polyglycolic acid, poly(lactide coglycolide), polyanhydrides, poly(methyl methacrylate), poly(ethylene-co-vinyl acetate), polysiloxanes, polymeric silica, latexes, dextran polymers, epoxies, polycarbonates, or combinations thereof. Conducting polymers and photoconductive materials can be used.

[00064] Substrates can be planar crystalline substrates such as silica based substrates (e.g. glass, quartz, or the like), or crystalline substrates used in, e.g., the semiconductor and microprocessor industries, such as silicon, gallium arsenide, indium doped GaN and the like, and includes semiconductor nanocrystals.

[00065] The substrate can take the form of a photodiode, an optoelectronic sensor such as an optoelectronic semiconductor chip or optoelectronic thin-film semiconductor, or a

biochip. The location(s) of the individual sensor polynucleotide(s) on the substrate can be addressable; this can be done in highly dense formats, and the location(s) can be microaddressable or nanoaddressable.

5 [00066] Silica aerogels can also be used as substrates, and can be prepared by methods known in the art. Aerogel substrates may be used as free standing substrates or as a surface coating for another substrate material.

10 [00067] The substrate can take any form and typically is a plate, slide, bead, pellet, disk, particle, microparticle, nanoparticle, strand, precipitate, optionally porous gel, sheets, tube, sphere, container, capillary, pad, slice, film, chip, multiwell plate or dish, optical fiber, etc. The substrate can be any form that is rigid or semi-rigid. The substrate may contain raised or depressed regions on which a sensor polynucleotide or other assay component is located. The surface of the substrate can be etched using well known techniques to provide for desired surface features, for example trenches, v-grooves, mesa structures, or the like.

15 [00068] Surfaces on the substrate can be composed of the same material as the substrate or can be made from a different material, and can be coupled to the substrate by chemical or physical means. Such coupled surfaces may be composed of any of a wide variety of materials, for example, polymers, plastics, resins, polysaccharides, silica or silica-based materials, carbon, metals, inorganic glasses, membranes, or any of the above-listed
20 substrate materials. The surface can be optically transparent and can have surface Si-OH functionalities, such as those found on silica surfaces.

25 [00069] The substrate and/or its optional surface are chosen to provide appropriate optical characteristics for the synthetic and/or detection methods used. The substrate and/or surface can be transparent to allow the exposure of the substrate by light applied from multiple directions. The substrate and/or surface may be provided with reflective "mirror" structures to increase the recovery of light.

[00070] The substrate and/or its surface is generally resistant to, or is treated to resist, the conditions to which it is to be exposed in use, and can be optionally treated to remove any resistant material after exposure to such conditions.

[00071] Sensor polynucleotides can be fabricated on or attached to the substrate by any suitable method, for example the methods described in U.S. Pat. No. 5,143,854, PCT Publ. No. WO 92/10092, U.S. Patent Application Ser. No. 07/624,120, filed Dec. 6, 1990 (now abandoned), Fodor et al., Science, 251: 767-777 (1991), and PCT Publ. No. WO 90/15070). Techniques for the synthesis of these arrays using mechanical synthesis strategies are described in, e.g., PCT Publication No. WO 93/09668 and U.S. Pat. No. 5,384,261.

[00072] Still further techniques include bead based techniques such as those described in PCT Appl. No. PCT/US93/04145 and pin based methods such as those described in U.S. Pat. No. 5,288,514.

[00073] Additional flow channel or spotting methods applicable to attachment of sensor polynucleotides to the substrate are described in U. S. Patent Application Ser. No. 07/980,523, filed Nov. 20, 1992, and U.S. Pat. No. 5,384,261. Reagents are delivered to the substrate by either (1) flowing within a channel defined on predefined regions or (2) "spotting" on predefined regions. A protective coating such as a hydrophilic or hydrophobic coating (depending upon the nature of the solvent) can be used over portions of the substrate to be protected, sometimes in combination with materials that facilitate wetting by the reactant solution in other regions. In this manner, the flowing solutions are further prevented from passing outside of their designated flow paths.

[00074] Typical dispensers include a micropipette optionally robotically controlled, an ink-jet printer, a series of tubes, a manifold, an array of pipettes, or the like so that various reagents can be delivered to the reaction regions sequentially or simultaneously.

EXCITATION AND DETECTION OF THE CHROMOPHORES

[00075] Any instrument that provides a wavelength that can excite the polycationic multichromophore and is shorter than the emission wavelength(s) to be detected can be used for excitation. The excitation source preferably does not significantly excite the signaling chromophore directly. The source may be: a broadband UV light source such as a deuterium lamp with an appropriate filter, the output of a white light source such as a xenon lamp or a deuterium lamp after passing through a monochromator to extract out

the desired wavelengths, a continuous wave (cw) gas laser, a solid state diode laser, or any of the pulsed lasers. The emitted light from the signaling chromophore can be detected through any suitable device or technique; many suitable approaches are known in the art. For example, a fluorimeter or spectrophotometer may be used to detect whether the test sample emits light of a wavelength characteristic of the signaling chromophore upon excitation of the multichromophore.

KITS

[00076] Kits comprising reagents useful for performing the methods of the invention are also provided. In one embodiment, a kit comprises a single-stranded sensor PNA that is complementary to a target polynucleotide of interest and a polycationic multichromophore. The sensor PNA is conjugated to a signaling chromophore. In the presence of the target polynucleotide in the sample, the sensor PNA is brought into proximity to the multichromophore upon hybridization to the target, which associates electrostatically with the polycationic multichromophore.

[00077] The components of the kit can be retained by a housing. Instructions for using the kit to perform a method of the invention can be provided with the housing, and can be provided in any fixed medium. The instructions may be located inside the housing or outside the housing, and may be printed on the interior or exterior of any surface forming the housing which renders the instructions legible. The kit may be in multiplex form, containing pluralities of one or more different sensor PNAs which can hybridize to corresponding different target polynucleotides.

EXAMPLES

[00078] The following examples are set forth so as to provide those of ordinary skill in the art with a complete description of how to make and use the present invention, and are not intended to limit the scope of what is regarded as the invention. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperature, etc.) but some experimental error and deviation should be accounted for. Unless otherwise indicated, parts are parts by weight, temperature is degree centigrade and pressure is at or near atmospheric, and all materials are commercially available.

[00079] Example 1. Identification of a Multichromophore/Signaling Chromophore Pair for FRET

[00080] The ability to transfer energy from the light harvesting multichromophore system to the signaling chromophore on a sensor PNA was demonstrated using the cationic water soluble conjugated polymer poly((9,9-bis(6'-N,N,N-trimethylammonium)-hexyl)-fluorene phenylene), polymer 1 with iodide counteranions prepared as described²³, and the sensor peptide nucleic acid PNA-C* having the sequence 5'-CAGTCCAGTGATACG-3' and conjugated to fluorescein (C*) at the 5' position. The respective absorption (green and orange) and emission (blue and red) spectra of polymer 1 and the sensor peptide nucleic acid PNA-C* are shown in Figure 2. Excitation was performed at 380 and 480 nm for 1 and PNA-C*, respectively. The data show that there is an optical window for the specific excitation of polymer 1. Moreover, there is excellent overlap between the emission of polymer 1 and the absorption of C* to allow FRET.³¹

[00081] Example 2. Demonstration of FRET in the Presence of Target Polynucleotide

[00082] The PNA-C* probe ([PNA-C*] = 2.5×10^{-8} M) was contacted with an equimolar amount of the complementary 15 base pair ssDNA, (5'-CGTATCACTGGACTG-3') 3, and in an identical fashion with a non-complementary 15 base ssDNA, (5'-ACTGACGATAGACTG-3') 4, in separate vessels in the absence of polymer 1. The annealing step was performed in the absence of buffer, *i.e.* at low ionic strength, at 2°C below the T_m of PNA-C* (72°C at 10^{-8} M, pH = 5.5).^{32,33} A melting experiment was performed and the absorbance monitored by UV/Vis spectroscopy at 260 nm.¹⁸ Increasing the temperature led to an increase in absorbance upon melting of the hybridized duplex in the sample containing the complementary ssDNA, as the two single strands absorb more highly than the hybridized duplex. As expected, the sample containing the non-complementary ssDNA did not show such an increase in absorbance, as no duplex was formed in that sample.

[00083] FRET was measured in annealed samples containing the complementary and non-complementary ssDNAs and polymer 1 ($[1] = 2.3 \times 10^{-7} \text{ M}$). The normalized emission spectra of PNA-C* in the presence of complementary (red) and non-complementary (black) DNA upon excitation of polymer 1 are shown in Figure 3. A FRET ratio > 11 times higher for the PNA/DNA hybrid was detected, relative to the non-complementary pair.³⁴ This difference in FRET demonstrates the specificity of the disclosed method for a given target polynucleotide. Furthermore, the fluorescein emission was more than 8 times larger than that obtained from direct C* excitation in the absence of 1.³⁵ This increased C* emission in the energy transfer complex comprising the sensor PNA, target polynucleotide and polycationic multichromophore indicates that optical amplification is provided by the multichromophore (polymer 1). This sensitized acceptor emission is demonstrated only in the presence of the complementary target polynucleotide.

15 [00084] Example 3. Optimization of Energy Transfer

[00085] Energy transfer was optimized by varying the ratio of compound 1 to PNA-C*. At a concentration of $[\text{PNA-C}^*] = 2.5 \times 10^{-8} \text{ M}$, initial additions of 1 caused an immediate rise in the FRET ratio. When $[1]$ far exceeded $[\text{PNA-C}^*]$, a decrease was observed. The maximum in the FRET ratio corresponds to a near 1:1 ratio of polymer chains to PNA strands, according to previously published molecular weight information.²³ Such a relationship was expected, since when $[1]/[\text{PNA-C}^*] < 1$, not all ssDNA/PNA-C* hybrid strands are complexed efficiently to independent polymer chains. Conversely, in the $[1]/[\text{PNA-C}^*] > 1$ regime, not all the photons harnessed by 1 (the donor) can be transferred to the DNA/PNA-C* hybrid (the acceptor). Note that the C* emission at the saturation point is more than 25 times greater than that obtained by direct C* excitation (480 nm), giving further evidence of signal amplification by the multiple chromophore structure of polymer 1.

[00086] Example 4. Use of an Organic Solvent to Decrease Background Signaling

[00087] Examination of Figure 3 shows a small fluorescein signal from the non-hybridized PNA probe, which may result from hydrophobic interactions between 1 and PNA-C*.³⁶ Addition of ethanol to the assay solution to a final concentration of 10% ethanol, under the identical conditions as the experiments shown in Example 2 resulted in a decrease in background C* emission. The presence of the organic solvent decreased hydrophobic interactions and reduced background C* emission by a factor of 3, at which point the background signal was almost undetectable using a standard fluorimeter.³⁷

[00088] Example 5. Target Polynucleotide Detection Using a Second Polycationic Multichromophore

[00089] The water soluble conjugated oligomer 2 prepared as described.²³ with an average $n=20$ was utilized as the light harvesting chromophore. The normalized emission spectra of PNA-C* in the presence of complementary (red) and non-complementary (black) DNA upon excitation of oligomer 2 in shown in Figure 4. The assay was performed as described in Example 2, with $[2]=6.7 \times 10^{-8}$ M and $[PNA-C*]=2.5 \times 10^{-8}$ M. Figure 4 shows C* emission was detected only when the complementary target polynucleotide was present. Comparison of Figures 3 and 4 demonstrates that use of conjugated polymers (oligomers) with higher molecular weights leads to higher FRET ratios. Thus, significantly higher FRET ratios and correspondingly higher sensitivities can be expected with polycationic multichromophores with higher molecular weights than those used in the examples.

[00090] Although the invention has been described in some detail with reference to the preferred embodiments, those of skill in the art will realize, in light of the teachings herein, that certain changes and modifications can be made without departing from the spirit and scope of the invention. Accordingly, the invention is limited only by the claims.

References

- ¹ Wang, J. *Nucleic Acid Res.* **2000** 28 3011.
- ² Umek, R.M.; Lin, S.W.; Vielmetter, J.; Terbruggen, R.H.; Irvine, B.; Yu, C.J.; Kayyem, J.F.; Yowanto, H.; Blackburn, G.F.; Farkas, D.H.; Chen, Y.P. *J. Mol. Diag.* **2001** 3 74.
- ³ Schork N.J.; Fallin D.; Lanchbury J.S. *Clini. Genet.* **2000** 58 250.
- ⁴ Balakin, K. V.; Korshun, V.A.; Mikhalev, I.I.; Maleev, G.V.; Malakhov A.D.; Prokhorenko, I.A.; Berlin, Yu.A. *Biosensors and Bioelectronics* **1998** 13 771.
- ⁵ LePecq, J.B.; Paoletti, C. *J. Mol. Biol.* **1967** 27 87.
- ⁶ Petty, J.T.; Bordelon, J.A.; Robertson, M.E. *J. Phys. Chem. B* **2000** 104 7221.
- ⁷ Cardullo, R.A.; Agrawal, S.; Flores, C.; Zamechnik, P.C.; Wolf, D.E. *Proc. Natl. Acad. Sci.* **1988** 85 8790.
- ⁸ Castro, A.; Williams, J.G.K. *Anal. Chem.* **1997** 69 3915.
- ⁹ Knemeyer, J.; Marmé, N.; Sauer, M. *Anal. Chem.* **2000** 72 3717.
- ¹⁰ Nielsen, P. E.; Egholm, M. *Peptide Nucleic Acids: Protocols and Applications*. Horizon Scientific Press, Portland, **1999**.
- ¹¹ Stender, H.; Fiandaca, M.; Hyldig-Nielsen, J.J.; Coull, J. *J. Microbiological Methods* **2002** 48 1.
- ¹² Egholm, M.; Buchardt, O.; Christensen, L.; Behrens, C.; Freier, S.M.; Driver, D.A.; Berg, R.H.; Kim, S.K.; Norden, B.; Nielsen, P.E. *Nature* **1993** 365 556.
- ¹³ Nielsen, P.E. *Current Opinions in Biotechnology* **1999** 10 71.
- ¹⁴ Demidov, V.V. *Biochem. Pharmacol.* **1994** 48 1310.
- ¹⁵ Egholm, M.; Nielsen, P.E.; Berg, R.H.; Buchardt, O.; Berg, R.H. *J. Am. Chem. Soc.* **1992** 114 9677.
- ¹⁶ Betts, L.; Josey, J.A.; Veal, J.M.; Jordan, S.R. *Science* **1995** 270 1838.
- ¹⁷ Hanvey, J.C.; Peffer, N.J.; Bisi, J.E.; Thomson, S.A.; Cadilla, R.; Josey, J.A.; Ricca, D.J.; Hassman, C.F.; Bonham, M.A.; Au, K.G.; Carter, S.G.; Bruckenstein, D.A.; Boyd, A.L.; Noble, S.A.; Babiss, L.E. *Science* **2002** 258 1481.
- ¹⁸ Tomac, S.; Sarkar, M.; Ratilainen, T.; Wittung, P.; Nielsen, P.E.; Nordén, B.; Gräslund, A. *J. Am. Chem. Soc.* **1996** 118 5544.
- ¹⁹ McQuade, D.T.; Pullen, A. E.; Swager, T.M. *Chem. Rev.* **2000** 100 2537.
- ²⁰ Chen, L.; McBranch, D.W.; Wang, H.-L.; Helgeson, R.; Wudl, F.; Whitten, D.G. *Proc. Natl. Acad. Sci. U.S.A.* **1999** 96 12287.
- ²¹ Dogariu, A.; Gupta, R.; Heeger, A.J.; Wang, H. *Synthetic Metals* **1999** 100 95.
- ²² Wang, J.; Wang, D.; Miller, E.K.; Moses, D.; Bazan, G.C.; Heeger, A.J. *Macromolecules* **2000** 33 5153.
- ²³ Stork, M.S.; Gaylord, B.S.; Heeger, A.J.; Bazan, G.C. *Adv. Mater.* **2002** 14 361. The molecular weight of polymer 1 was determined to be 8,600 g/mole (M_n).
- ²⁴ Kabanov, A.V.; Felgner, P.; Seymour, L.W., Eds. *Self-Assembling Complexes for Gene Delivery. From Laboratory to Clinical Trial*; John Wiley: Chichester, 1998.
- ²⁵ Kircheis, R.; Blessing, T.; Brunner, S.; Wightman, L.; Wagner, E. *J. Controlled Release* **2001** 72 165.
- ²⁶ Wolfert, M.A.; Dash, P.R.; Navarova, O.; Oupicky, D.; Seymour, L.W.; Smart, S.; Strohal, J.; Ulbrich, K. *Bioconjugate Chem.* **1999** 10 993.
- ²⁷ Ganachaud, F.; Elaissari, A.; Pichot, C.; Laayoun, A.; Cros, P. *Langmuir* **1997** 13 701.
- ²⁸ Smith, J.O.; Olson, D.A.; Armitage, B.A. *J. Am. Chem. Soc.* **1999** 121 2628.
- ²⁹ Harada, A.; Kataoka, K. *Science* **1999** 283 65.
- ³⁰ Bronich, T.K.; Nguyen, H.K.; Eisenberg, A.; Kabanov, A.V. *J. Am. Chem. Soc.* **2000** 122 8339.
- ³¹ Lakowicz, J.R. *Principles of Fluorescence Spectroscopy*. Kluwer Academic/Plenum Publishers: New York, **1999**.
- ³² Giesen, U.; Dleider, W.; Berding, C.; Geiger, A.; Ørum, H.; Nielsen, P.E. *Nucleic Acids Res.* **1998** 26 5004.
- ³³ An interactive version of reference 28 can be found at applied biosystems custom probe designer web site: www.appliedbiosystems.com/cgi-bin/calculator/ab_configured/oligodesigner/designer.cgi
- ³⁴ The FRET ratio is defined as the integrated acceptor emission over the integrated emission of the donor.
- ³⁵ Fluorescein at pH=5.5 is not in its high quantum yield dianionic form, thus we would expect higher C* emission at higher pH, but at the expense of charge neutrality on the PNA-C* complex.
- ³⁶ Gaylord, B.S.; Wang, S.; Heeger, A.J.; Bazan, G.C. *J. Am. Chem. Soc.* **2001** 123 6417.
- ³⁷ PTI Quantum Master fluorimeter equipped with a Xenon lamp excitation source and a Hamamatsu PMT.